How do you see CG?

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How Do You See CG?

Minireview

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The mammalian immune response to microbial pathogens relies on both innate and adaptive components (Hoffmann et al., 1999). The immediate, innate response is mediated largely by white blood cells such as neutrophils and macrophages, cells that phagocytose and kill the pathogens, and that concurrently coordinate additional host responses by synthesizing a wide range of inflammatory mediators and cytokines (Aderem and Underhill, 1999). In macrophages, the infectious agent is killed and degraded within the maturing phagosome, and components of the pathogen are presented to T cells, resulting in the activation of the adaptive immune response and the establishment of protective immunity (Aderem and Underhill, 1999). A major challenge to the innate immune system is the discrimination of a large number of potential pathogens from self. This challenge has been met by the evolution of a variety of receptors that recognize conserved motifs on pathogens that are not found in higher eukaryotes; these so called pattern-recognition receptors recognize pathogen-associated molecular patterns (Janeway and Medzhitov, 1998).

Every organism, even bacteria, have mechanisms of innate host defense to protect against invasion by potential pathogens. The major defense mechanism employed by bacteria relies on recognition of foreign DNA. Bacteria methylate specific sequences in their own genome and produce restriction enzymes that cleave unmethylated sequences in the DNA of an invading pathogen such as a bacteriophage (Murray, 2000). Only quite recently have we come to recognize that specialized immune cells in mammals can employ a very similar mechanism. The CpG dinucleotide in particular sequence contexts (PuPuCGPyPy) is greatly reduced in frequency in the mammalian genome, and where it occurs, the cytosine commonly is methylated. DNA containing unmethylated CG motifs is able to activate murine myeloid cells (macrophages and dendritic cells) and B lymphocytes, a response that stimulates immune defenses that may be desirable in a therapeutic or immunoprophylactic context (Sester et al., 1999; Wagner, 1999, Chu et al., 2000; Hemmi et al., 2000; Krieg and Wagner, 2000, and references therein). Among native foreign DNAs tested, bacterial DNA is the most active immunomodulator, but mammalian cells also can recognize and respond to yeast, insect, and nematode genomic DNA while ignoring high concentrations of their own DNA, even when it is demethylated (Sun et al., 1997). Activation of immune cells by foreign DNA appears to require uptake into the cell by receptor-mediated endocytosis and endosome acidification, suggesting that the recognition structure is either endosome-associated or cytoplasmic (Krieg and Wagner, 2000). Therapeutic applications of the response to foreign DNA have been expedited by the observation that short, CG-containing oligonucleotides (CpG-ODN) retain activity, even when stabilized with noncleavable phosphorothioate bonds (Krieg and Wagner, 2000). The remarkable specificity of the response is seen in the observation that reversal of the CG motif to GC in active CpG-ODN is sufficient to abolish activity. Aside from the core dinucleotide, variation in flanking bases alters the dose response curve for activation, for example a 5′ C residue (a CCG core) reduces the efficacy (Sester, et al., 1999, Krieg and Wagner, 2000). Such structure–function studies imply the existence of a recognition protein that can bind to either single-stranded or double-stranded DNA and that it has exquisite specificity for the CG core in an optimal context. The nature of that recognition molecule has thus far eluded identification. Two recent papers (Chu et al., 2000; Hemmi et al., 2000) promise a rapid resolution of this issue, but the connection between their conclusions is not immediately apparent.

Toll-like Receptor 9 and Components of Its Signaling Pathway Are Required for the Response to CpG-DNA

The study of Akira and colleagues (Hemmi et al., 2000) builds on parallels between the recognition of foreign DNA and other microbial products. The Toll-like receptors, first identified in *Drosophila*, have been implicated as the receptors that enable the innate immune system to recognize classes of molecules that are common to pathogenic microorganisms but are absent or underrepresented in the host, the so-called pathogen associated molecular patterns (Aderem and Ulevitch, 2000). In mammals, TLR4 is required for the immune response to bacterial lipopolysaccharide (LPS), the major cell wall component of Gram-negative bacteria, while TLR2 is required for the response to several components of Gram-positive organisms, including lipopeptides and peptidoglycan as well as yeast particles (Aderem and Ulevitch, 2000). Different Toll-like receptors can also combine to extend their repertoire of detection; for example TLR2 can combine with TLR6 to detect peptidoglycan, whereas TLR2 appears to combine with a yet unknown TLR to detect lipopeptide (Ozinsky et al., 2000).

The mechanism by which TLRs stimulate the production of inflammatory molecules is being clarified (Figure 1) (Janeway and Medzhitov, 1998; Aderem and Ulevitch, 2000). Ligation of a TLR promotes dimerization and results in the recruitment of MyD88, an adaptor protein containing two domains: a C-terminal TIR domain that interacts with the TIR domain of the receptor, and an N-terminal death domain. This death domain undergoes homophilic interaction with the death domain of a serine/threonine protein kinase known as IRAK; this leads to the autophosphorylation of IRAK. Autophosphorylated

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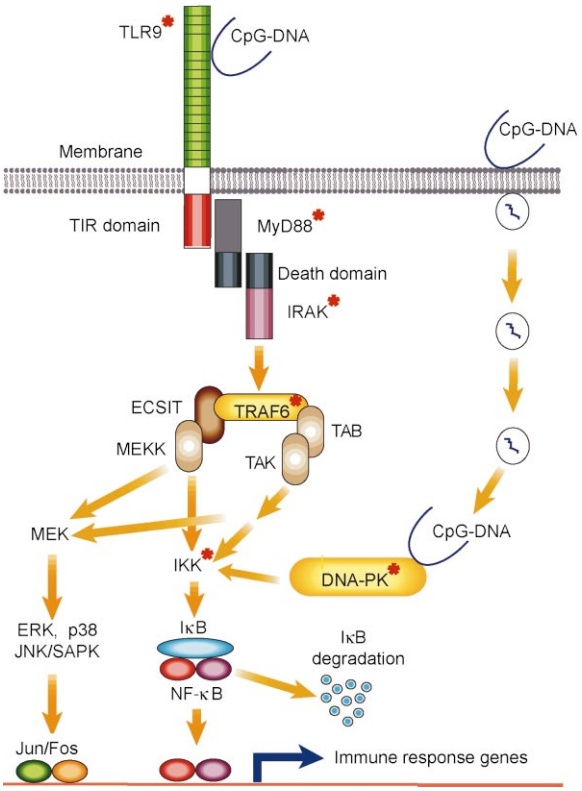

Figure 1. Signaling Pathways through which TLR9 and DNA-PK Might Activate the Transcription of Immune Response Genes

TLR9 has an intracellular domain that is homologous with that of the IL-1 receptor, and is known as TIR. TIR binds to a homologous domain in an adaptor protein MyD88, which also contains a death domain; this interacts with the death domain in the serine kinase IRAK. IRAK interacts with an adaptor protein known as TRAF6. TRAF6 links to the MAP3 kinase TAK-1, through an adaptor TAB2. TAK-1 is involved in the activation of the transcription factor NF-κB through the activation of IκB kinases, and in the activation of the AP-1 transcription family members Jun and Fos, by way of additional MAP kinases. Both AP-1 transcription family members and NF-κB are required for the transcription of immune response genes. TRAF6 is known to act through more than one pathway. For example, the adaptor ECSIT bridges TRAF6 to the MAP 3-kinase MEKK-1. DNA-PK can activate NF-κB through the activation of IκB kinases. See the text for a more complete description of the signaling pathway. It is not clear how CpG-DNA activates either the TLR9 or the DNA-PK pathway; it could do so directly or indirectly. Deletion of the genes encoding the proteins marked with asterisks results in mice that are unable to respond to CpG-DNA. CpG-DNA may be endocytosed prior to activation of DNA-PK, although how it crosses the endosomal membrane is unknown.

IRAK then forms a complex with TRAF6 and this, in turn, results in the oligomerization of TRAF6. At this point, the details of the pathway become less clear. Somehow the oligomerization of TRAF6 activates TAK-1, a member of the MAP 3-kinase family, and this leads to the activation of the family of IκB kinases. These kinases, in turn, phosphorylate IκB, leading to its proteolytic degradation and the translocation of NF-κB to the nucleus. Concomitantly, members of the activator protein-1 (AP-1) transcription factor family, Jun and Fos, are activated, and both AP-1 transcription factors and NF-κB are required for cytokine production. Although gene deletion studies have supported this activation pathway, there is clearly substantial additional complexity. For example, many components of the signaling pathway have homologs: at least three IRAK homologs have been demonstrated, and these are known to compensate partly for each other.

Akira and coworkers (Hemmi et al., 2000) deleted the gene encoding another member of the TLR family, TLR9, in mice. The mutation of the TLR9 gene produced viable mice, but B cells, macrophages, and dendritic cells from these animals failed to respond to activation by a stimulatory CpG-DNA, and the mice were resistant to toxic shock elicited by CpG-DNA in vivo. By contrast, responses of the myeloid and lymphoid cells to TLR4 (LPS) and TLR2 agonists (peptidoglycan) were retained. The phenotype of the TLR9 knockout is consistent with earlier evidence showing that the common Toll signaling pathway, involving adaptor protein MyD88, downstream effectors TRAF-6 and IRAK, and ultimately transcription factor NF-κB, was activated by foreign DNA, and that the MyD88 "knockout" was unresponsive to foreign DNA (Hacker et al., 2000, Schnare et al., 2000). In addition, consistent with evidence that uptake of CpG-DNA is required for its immune stimulatory activity, Hemmi et al. (2000) cite unpublished evidence that MyD88 colocalizes with vesicles containing CpG-DNA. A relationship between the endocytic pathway and TLR signaling also is suggested by the observation that TLR1, 2, and 6 are recruited to phagosomes in macrophages (Aderem and Ulevitch, 2000; Ozinsky et al., 2000).

DNA-Dependent Protein Kinase Is Required for Host Response to CpG-DNA

The paper of Raz and colleagues (Chu et al., 2000) builds upon a quite different perspective of the nature of the response to foreign DNA. During the normal cell cycle, mammalian cells scan their own genomes for damage. The damage detection and repair pathways are linked by a signaling mechanism designed to stop the cell cycle for sufficient time to permit the mutation to be corrected. Depending upon the nature and extent (repairability) of the damage, either antiapoptotic or proapoptotic pathways can be activated. The response of immune cells to foreign DNA has some parallels with the DNA damage response (Sester et al. 1999). In proliferating macrophages, foreign DNA causes cell cycle arrest, but is antiapoptotic (Sester et al., 2000). Similar anti-apoptotic effects in B cells have been analyzed extensively (Yi and Krieg, 1998) although in this lineage foreign DNA can also be mitogenic. The signaling pathway following DNA damage commonly involves a key cell cycle regulator, the transcription factor p53. One of the major components of the DNA damage response is DNA-dependent protein kinase (DNA-PK), which can phosphorylate p53, leading to increased transcriptional activation of genes involved in cell cycle arrest (Dumaz and Meek, 1999). This enzyme, a member of the phosphatidyl-inositol-3 kinase family, is activated following recognition of double-stranded DNA breaks (ends) by the Ku DNA binding proteins. Recent evidence indicates that DNA-PK can also bind single-stranded DNA ends (Hammarsten et al. 2000), but sequence-specific DNA recognition had not previously been reported. Chu et al. (2000) show that immunostimulatory CpG-DNA trigger DNA-PK activation (as measured by p53 kinase activity)

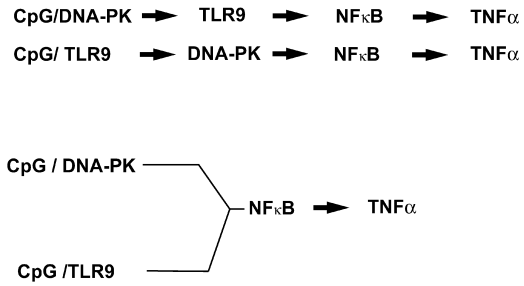

Figure 2. TLR9 and DNA-PK May Be Activated Sequentially or in Parallel by CpG-DNA

In a linear model of activation, DNA-PK may be either upstream or downstream of TLR9. In the parallel model, both DNA-PK and TLR9 signals are required for NF-κB-dependent transcription of the TNFα gene. See the text for details.

in either affinity-purified enzyme preparations or intact cells. They establish a plausible signaling pathway, by showing that the activated DNA-PK enzyme can also phosphorylate IκB-kinaseβ, leading to the activation of the key transcription factor NFκB, which is, in turn, connected to transcriptional activation of the production of many inflammatory cytokine genes (Figure 1). Null mutations in either the catalytic subunit of DNA-PK or in the IκB-kinaseβ gene were found to selectively impair the ability of the mice, or their macrophages, to respond to stimulatory CpG-DNA (or bacterial DNA) with inducible cytokine gene expression. By contrast, a mutation in a related enzyme, ataxia telangectasia-mutated (ATM), which also participates in recognition of some types of DNA damage, had no effect on the response to immunostimulatory DNA.

Mechanisms by which TLR9 and DNA-PK Might Interact

It is intriguing that two groups simultaneously present data implicating two apparently unlinked biological pathways in CpG-DNA detection (Figure 1). A number of possible models could explain the data, although no single one of these models presently is favored. First, it is possible that DNA-PK and TLR9 provide parallel signals that are both required for innate immune activation. In this model both pathways lead to NFκB activation as described in Figure 1. The weakness of this model is that the TLR9 pathway is derived by analogy with the TLR4 pathway, and that DNA-PK is not required for activation of NFκB by TLR4 (as implied by the lack of any effect of the mutation of the enzyme on the response of macrophages to LPS). However, since we have an incomplete understanding of the TLR4 pathway, it is possible that DNA-PK serves a function in TLR9 signaling that is analogous to an, as yet, undefined component in the TLR4 pathway. This suggests a second model where TLR9 and DNA-PK are sequentially activated in a linear signaling pathway (Figure 2). In this model TLR9 could mediate the recognition of CpG-DNA, and DNA-PK could be part of its downstream signaling pathway. Alternatively, DNA-PK could mediate CpG-DNA recognition and activate a downstream signaling pathway that requires TLR9. For example, DNA-PK could stimulate the production of a secreted TLR9 ligand that functions in an autocrine manner. This would be analogous to Toll receptor activation by the endogenous ligand, spaetzle, in *Drosophila* (Hoffmann et al., 1999; Aderem and Ulevitch, 2000). To decide between these models it will be important to determine whether TLR9 or DNA-PK mediate specific sequence recognition of CpG-DNA.

The effects of null mutations in mice always need to be interpreted with caution, and it remains possible that neither of these molecules is the specific CpG-DNA receptor. There is no obvious structural basis for the recognition of DNA by the extracellular or intracellular domains of TLR9, and no evidence is presented by Hemmi et al. (2000) to show that TLR9 binds DNA directly or is functionally altered following DNA recognition. These authors examine only phosphorothioate-modified oligonucleotides. There is evidence that the phosphorothioate backbone is recognized independently by macrophages and amplifies some DNA response pathways while inhibiting others (Sester et al. 2000). Hence, TLR9 could be a receptor for phosphorothioates, rather more consistent with the kind of ligands bound by other TLRs. As noted by the authors (Chu et al., 2000), the sequence specificity of DNA-PK is also not clearly correlated with biological activity. Sheared mammalian DNA, or double-stranded non-CpG oligonucleotides, would both activate this enzyme, yet neither mimics the response to bacterial DNA or CpG oligonucleotides. It is therefore possible that additional molecules contribute in providing greater molecular discrimination. Furthermore, previous studies have shown that a mutation in the DNA-PKcs gene (the catalytic subunit of DNA-PK) in mice with severe combined immunodeficiency (SCID) had no effect on response to CpG DNA (Chace et al. 1997). Although the SCID is not a complete null mutation of DNA-PK, it clearly has a powerful phenotypic effect. Recent studies have confirmed that the C-terminal domain of DNA-PKcs, which is missing in the SCID mouse, is actually needed for catalytic activity (Beamish et al., 2000), implying that the kinase activity of DNA-PKcs may not be required for CpG induced signaling. A resolution of the difference in phenotype between the SCID, and the DNA-PKcs knockout, may give an insight into the precise role of this gene product.

An additional element that needs to be reconciled with the models described above is the requirement for endocytosis in the CpG-DNA response; obviously this adds considerable complexity. Since we do not yet know the localization of TLR9, it is possible that ligand must be delivered to the receptor via an acidified endocytic compartment. Alternatively, endocytosis may be required for TLR9 signaling. Finally, if DNA-PK is the receptor for CpG-DNA, its localization within the nucleus and cytoplasm would require delivery of the ligand to the cytoplasm, a process that might require prior endocytosis (Figure 1). Clearly, much remains to be learned about this important pathway.

Other questions also come to mind. The deletion of either DNA-PK or TLR9 might have additional consequences that may indirectly affect the CpG-DNA recognition pathway. For example, DNA-PK has a central role in the generation of immunoglobulin and T cell receptor rearrangements, and given the known interactions between innate and adaptive immunity, indirect regulation of the CpG-DNA response might also occur. Additionally, the innate response to CpG-DNA is restricted to

host immune cells, whereas DNA-PK has a much wider distribution. Regardless of these questions, there is no doubt that the very important contributions by the groups of Akira and Raz represent a big boost to the study of the actions of foreign DNA and will generate new avenues to identify immunomodulators that mimic the actions of foreign DNA, but are more bioavailable.

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